

# Variable rewards and discrimination ability in an insect herbivore: what and how does a hungry locust learn?

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## Summary

With the exception of honeybees, there have been few good invertebrate models for associative learning. Grasshoppers and locusts (Orthoptera: Acrididae) possess a number of characteristics that make them excellent candidates for such studies, and in this paper we present a novel protocol, based on a Y-maze, that is specifically designed for studying their learning and choice behaviour. Three separate experiments were conducted using individual gregarious forms of the desert locust, *Schistocerca gregaria*. In our first experiment, coloured arms of a two-sided Y-maze provided a large or small amount of wheat for nine choice-trials. In the second experiment, locusts discriminated odours with wheat rewards for nine choice-trials. The odour–wheat reward combinations were then reversed for an additional nine

choice-trials. For the third experiment, the locusts again discriminated odours, but here we used artificial foods and the rewards differed in their concentration of protein and digestible carbohydrate. The results indicate that, in addition to showing good acquisition of choice performance, the locusts also took less time to reach the larger-rewarded option. The data indicate that our protocol is highly sensitive for recording choice behaviour in acridids and reveals the potential they have for advancing our current understanding of associative learning and the field of learning in general.

Key words: associative learning, choice behaviour, insect learning, locust, *Schistocerca gregaria*.

## Introduction

Invertebrates, particularly insects, are useful organisms for the study of associative learning because they have relatively simple nervous systems and can be reared and maintained in large numbers with relative ease. Honeybees, for example, have brains containing around 960 000 neurons (Menzel and Giurfa, 2001), and because they live in colonies of 10 000–60 000, test subjects are usually plentiful. These factors, combined with the fact that honeybees can be trained to perform tasks that reflect learning (e.g. the proboscis extension reflex, landing on coloured or scented targets), explain why more is currently known about associative learning in honeybees than in any other invertebrate species (see reviews by Bitterman, 1996). We advocate, however, that the field of associative learning would benefit from a more parametric study of a new invertebrate model organism, and in this paper we promote the use of grasshoppers and locusts (Orthoptera: Acrididae).

Acridids, like honeybees, exhibit both aversion (e.g. Lee and Bernays, 1988, 1990; Champagne and Bernays, 1991; Behmer et al., 1999) and appetitive learning (e.g. Bernays and Wrubel, 1985; Simpson and White, 1990; Raubenheimer and Tucker, 1997) and have highly developed sensory capacities and motor

skills (see Uvarov, 1966; Chapman and Joern, 1990; Burrows, 1996; Chapman, 2003), which have been examined in great detail (reviewed by Burrows, 1996). Acridids, however, offer some unique advantages over honeybees. For instance, their nutritional physiology has been investigated in great detail (reviewed by Simpson and Raubenheimer, 2000), which permits a broader interpretation of learned behaviour related to food acquisition, and they readily eat synthetic foods, which allows for the testing of nutrient-specific appetitive learning. They are also hemimetabolous, which allows for testing both within and across developmental stages, and since they are diverse, opportunities exist to explore how natural-history traits (e.g. specialist vs generalist feeders, solitary vs gregarious individuals) influence learning abilities.

In this paper, we present a novel protocol specifically designed for acridids that allows learning behaviour, as recorded by changes in choice as well as response latency, to be measured. Response speed, although a common measure of learning in vertebrates, has not proved a sensitive measure within invertebrate models and has yet to be evaluated in acridids. This paper describes experiments using gregarious desert locusts (*Schistocerca gregaria*) run in a specially

designed two-sided Y-maze with arms discriminated by colour or odour and rewards differing in amount of fresh wheat grass or artificial diets with different concentrations of protein and carbohydrate.

## Methods and Results

### *Insects*

Desert locusts, *Schistocerca gregaria* (Forskål), came from crowd-reared (gregarious) cultures that have been kept in the Department of Zoology, Oxford, UK since 1983. The locusts were fed a diet of greenhouse-grown seedling wheat and wheat germ and maintained at 29–31°C under a 12 h:12 h L:D light regime. Female nymphs were collected from the culture immediately upon moulting to the fifth (final larval stage) stadium (day 0). They were then transferred to individual arenas (8×14×6 cm), where they were fed and observed for 2 days, before being used in experiments (day 3). Each set of experiments had 12 replicates, and insects were prevented from visually interacting with one another throughout the course of our experiments.

Before proceeding to our first experiment, a brief note concerning the structure of this section is warranted. For each experiment, we present methods followed immediately by the results.

### *Experiment 1: testing colour and amount discrimination*

#### *Identifying test subjects*

To standardise nutritional state and levels of motivation in our experimental test subjects, a selection protocol was employed. Prior to each individual test, an initial group of 10 newly moulted locusts was collected (day 0) and fed seedling

wheat and wheat germ until the early evening of day 1. On the morning of day 2, usually around 09.00 h, each of the 10 locusts was given a small piece of wheat (approximately 2–3 mg), followed by another similarly sized piece 1 h later (10.00 h). We recorded the time at which this second piece was eaten, and then 5 min later presented the locusts with another small piece of wheat. This pattern was followed until at least two individuals had eaten 10 pieces of wheat. These two locusts were then given food *ad libitum* until 16.00 h, and all other locusts were returned to the rearing culture. On the morning of day 3, at approximately 09.00 h, our two test locusts were given a small piece of wheat (2–3 mg), and we recorded when it was eaten. Approximately 1 h later, a second piece of wheat was presented to each locust. Of these two locusts, we selected for testing the one that was first to eat both pieces of wheat. In the case of a tie, we selected the one that ate more quickly during the previous day. Our test insect was then transferred to our experimental test arena.

### *Experimental arena and viewing platform*

The test arena, a two-sided Y-maze (Fig. 1), was made of clear Plexiglas and had four removable 'arms'. Within the central region of the arena we placed six 'gates'. Four of these permitted us to allow and/or deny access to each arm of the arena, while the other two were used to confine the animal to the central region of the test arena. The outside walls of the central chamber were wrapped in white tape, except for a small window (7×5 cm) on the side facing the observer, which was used for viewing the insect. The white tape stopped the test insect from seeing the arms through the walls of the test arena; in addition, it reduced interactions with the person doing the observations. The top of the arena remained clear, which

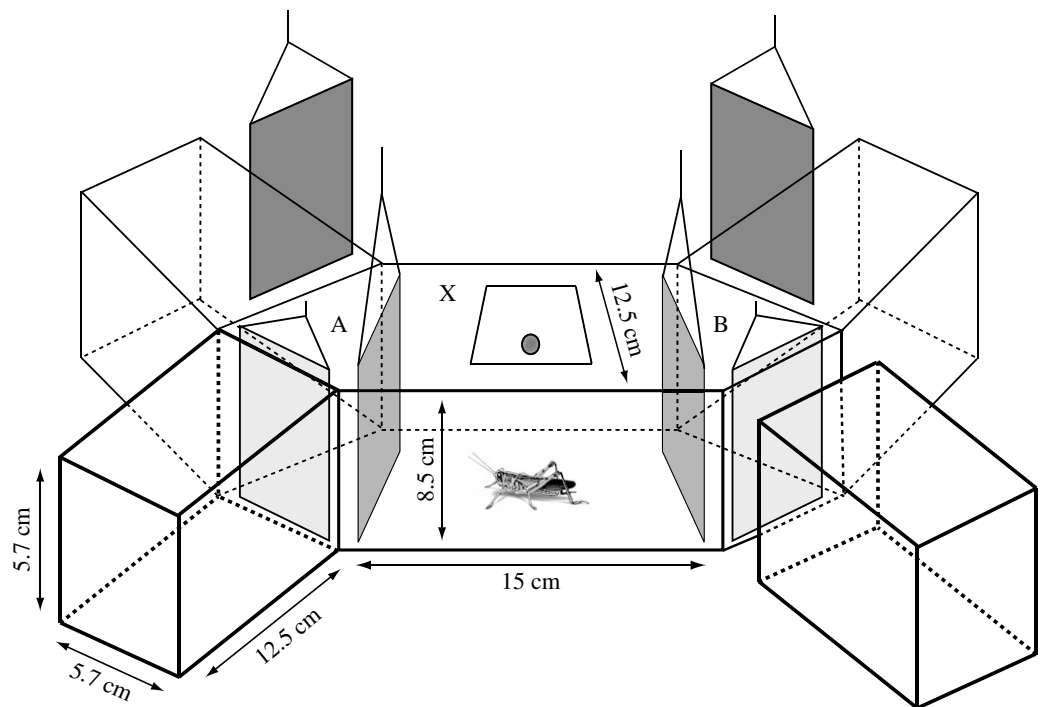


Fig. 1. Diagram of the two-sided Y-maze. Moveable gates separate area X from areas A and B. Gates also separate areas A and B from the removable arms.

exposed the animal to light while also allowing the observer to view the test insect.

The arena itself was placed inside a larger platform that had sheets of white bench-coating paper covering both the sides and back. Extending down the front of the arena was a white cloth sheet, containing a small viewing window (17×9 cm). Two fluorescent lights (Sunglo 20 W, 24/58.98 cm, 1230 lumens, 125 lux; Rolf C. Hagen, Inc.; <http://www.hagen.com/uk/>) ran lengthwise inside the top of the platform, which ensured that light spread evenly within the platform (measured at 242 lux, using a TES 1330A Digital Lux Meter; <http://www.tes.com.tw/>). Finally, a pulley system was erected across the top of the platform, with colour-coded handles running out from both sides of the viewing platform, which allowed us to raise and lower all six gates independently of one another.

#### Arms and rewards

We created two different coloured arms by wrapping the Plexiglas sleeves (12.5×5.7×5.7 cm) in cellophane paper (green or yellow), and, in doing so, special care was taken to match their relative luminance (green=164 lux; yellow=182 lux). Inside each arm, near their centres, was a small, moistened piece of cotton wool, on top of which we placed either one or four pieces of wheat (each piece approximately 5 mm in length). Henceforth, we categorise large rewards as being 'positive' and small rewards as being 'negative' (negative implying less quantity or quality, not the aversive nature of the reinforcement). The wheat was placed on the cotton wool so that it faced away from the entrance and could not be seen until the locust was inside an arm. Over the course of an experiment, the number of wheat pieces associated with a particular colour always remained constant, but we balanced the reward size/colour combination across animals.

#### Behavioural observations

New test insects were always placed in the centre of the arena (with all gates down) and left for 1 h to acclimate to the new environment. After 1 h, one green and one yellow arm containing wheat were inserted into both sides of the arena, and the gates allowing access to these arms were raised, but only on one side of the arena. Next, we raised the larger gate that was adjacent to these two arms. By opening the gates in this order, we insured that the locust was making its choice from a central point of the arena, which lay some distance from the two arms. At this time, we also began recording the behaviour and movement of the locust, using the software package The Observer 3.0 (Noldus Information Technology, Inc., Wageningen, The Netherlands). Among the events we recorded were the time at which a locust entered its first arm (henceforth referred to as 'enter time') and the time from entering the arm to the point at which it began to eat the food ('approach time'). Once the locust began to feed, we lowered the gate of the alternative arm. When feeding ended, we then recorded the amount of time it took for the locust to leave the

arm ('exit time'). Immediately after leaving the arm, we lowered its gate, and, after the locust had returned to the middle of the arena, we raised the gate for the other arm. Next, we recorded the enter time, approach time and exit time for the second arm. By conducting each trial in this manner, we ensured equal experience with both options during training. Upon leaving the second arm, the gate for that arm was lowered and we left the locust in the middle region for a period of 5 min. A second choice, followed by forced exposure to the alternative, started when we opened the opposite three gates, as described above. During the next 5-min rest period, the arms were removed and new clean arms, containing fresh wheat, were attached. In total, we repeated this sequence nine times for each subject (nine choices, and nine experiences with the rewards associated with each coloured arm).

Upon completion of training, we allowed the locust to rest in the central region for 5 min before conducting a non-reinforced preference test (resistance to extinction test). For this test, there was no food in any of the arms, and we raised all of the gates simultaneously, so that the locust had access to all four arms (two green, two yellow). We recorded, over a 20-min period, which arms were entered and for how long locusts stayed in a particular arm. We also recorded the combined amount of time locusts spent in the middle region of the arena.

#### Results

The measured performance of locusts, plotted in terms of the proportion of choices for the colour associated with the larger reward for each of the nine choice-runs, is shown in Fig. 2. The

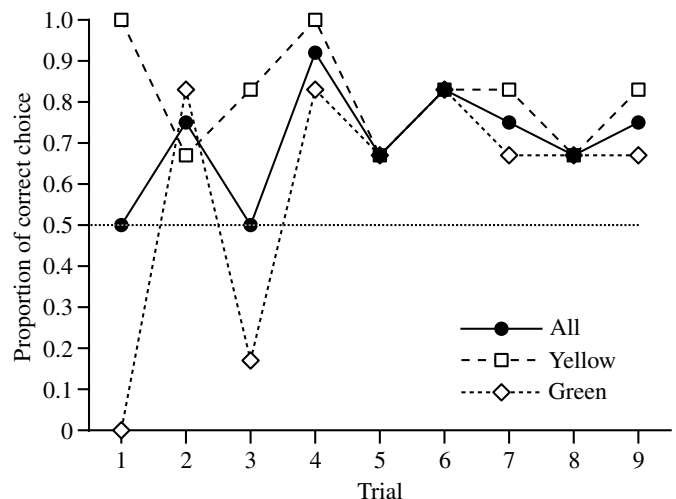


Fig. 2. Choice data, expressed as a proportion, for each of nine successive choice-trials. Naïve locusts were allowed to choose between two coloured arms (green or yellow), one containing four pieces of wheat, the other containing one piece of wheat. A total of 12 locusts were observed, and for each locust the colour/reward combination remained constant over the entire experiment (six locusts had the high reward paired with green, while six had the high reward paired with yellow). Data are also presented for the positive rewards combined. The dotted line indicates indifference to the positive and negative rewards.

first point to make is that naïve locusts (Trial 1) always selected the yellow arms. Insects often show an inherent bias for yellow (e.g. Bernays and Wrubel, 1985; Holliday and Holliday, 1995; Weiss, 1997), so this result is not necessarily surprising. Regardless of what drives the yellow-bias, our results indicate that this bias is overcome rather quickly. Analysis using a *t*-test with an expected value of 0.5 (indifference) revealed that locusts in the green-positive trials had a significant preference (greater than 0.5) for the green arms over all nine trials [ $t_{0.05(1),5}=3.05$ ,  $P=0.014$ ] and that, on average ( $\pm$  S.E.M.), green-positive arms were selected first  $61.3\pm3.7\%$  of the time. Preference for green-positive arms is even stronger if Trial 1 is excluded (thus eliminating the initial bias for yellow); selection increases to  $68.8\pm4.3\%$ , as does the significance level [ $t_{0.05(1),5}=4.392$ ,  $P=0.007$ ]. For locusts on the yellow-positive treatment, there was a significant preference for yellow over the entire training period [ $t_{0.05(1),5}=8.63$ ,  $P<0.001$ ], with yellow-positive arms being selected first, on average,  $81.7\pm4.1\%$  of the time. If Trial 1 is again excluded, the results do not change; yellow arms are selected over green arms  $79.2\pm4.2\%$  of the time, and this preference is highly significant [ $t_{0.05(1),5}=7.00$ ,  $P<0.001$ ]. When the effect of colour (yellow or green) was examined over all nine trials, collapsed across reward amount, a significant preference for yellow over green was observed [ $t_{0.05(2),10}=3.90$ ,  $P=0.003$ ]. However, if we exclude Trial 1 (to remove the effect of the colour bias), the effect of colour was no longer significant [ $t_{0.05(2),10}=1.75$ ,  $P=0.111$ ].

In addition to choice behaviour, we were also concerned with the latency of three activities: enter time, approach time and exit time. To test for differences in response latency (1) over the course of training, (2) between the positive and negative stimuli and (3) between the two colours, we used a repeated-measures analysis (RMA) where trials and stimulus were treated as within-subject factors and colour was treated as a between-subject factor (SPSS version 11.5; SPSS Inc., Chicago, IL, USA). When the assumption of sphericity was violated (epsilon value  $>0.70$ ), we employed the Huynh-Feldt correction procedure. For each latency measurement, data from all nine choice-trials were analysed.

We first examined the mean natural log latency of the entering time for the positive and negative colour and found a significant trials effect ( $F_{8,80}=2.84$ ,  $P=0.008$ ). As seen in Fig. 3A, the trend was for locusts to enter the arms more quickly over the course of training. We did not, however, detect a trials  $\times$  colour ( $F_{8,80}=1.15$ ,  $P=0.342$ ) or trials  $\times$  stimulus interaction ( $F_{8,80}=1.20$ ,  $P=0.307$ ). We did, however, find a significant stimulus  $\times$  colour interaction ( $F_{1,10}=6.41$ ,  $P=0.030$ ). The data showed that locusts entered yellow-positive arms much faster than yellow-negative arms, but that entry times into green arms were similar regardless of the reward size.

We also found a significant trials effect for approach time ( $F_{8,80}=3.14$ ,  $P=0.004$ ), with locusts showing reduced latencies over the course of the experiment (Fig. 3B). Approach time was also sensitive to the positive and negative stimulus

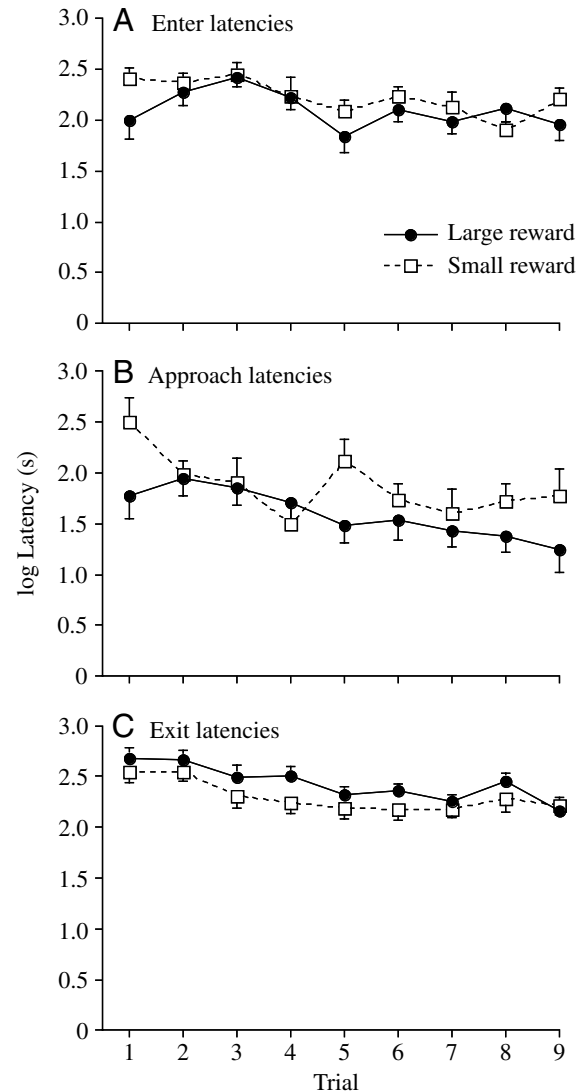


Fig. 3. The mean ( $\pm$  S.E.M.) natural log latency of response for the positive and negative colours over the course of training. Three measurements were recorded: (A) enter time, (B) approach time and (C) exit time.

( $F_{1,10}=5.30$ ,  $P=0.044$ ), with locusts taking less time to reach the food in the positive arm compared with the negative arm. This metric was not affected by an interaction between trials and stimulus ( $F_{8,80}=1.25$ ,  $P=0.283$ ) nor was there a significant colour effect ( $F_{1,10}=0.45$ ,  $P=0.517$ ) or stimulus  $\times$  colour interaction ( $F_{1,10}=0.01$ ,  $P=0.996$ ).

The final latency measure, exit time, also showed a significant trials effect ( $F_{8,80}=5.57$ ,  $P<0.001$ ), with locusts staying in the arms for shorter lengths of time after they completed feeding (Fig. 3C). We also detected a significant stimulus effect ( $F_{1,10}=15.86$ ,  $P=0.003$ ), with locusts remaining in the positive arms longer than the negative arms, but no significant stimulus  $\times$  trials interaction was observed ( $F_{8,80}=0.34$ ,  $P=0.950$ ). There was a significant stimulus  $\times$  colour interaction ( $F_{1,10}=16.01$ ,  $P<0.001$ ). For locusts in green arms, the exit time was similar regardless of the stimulus

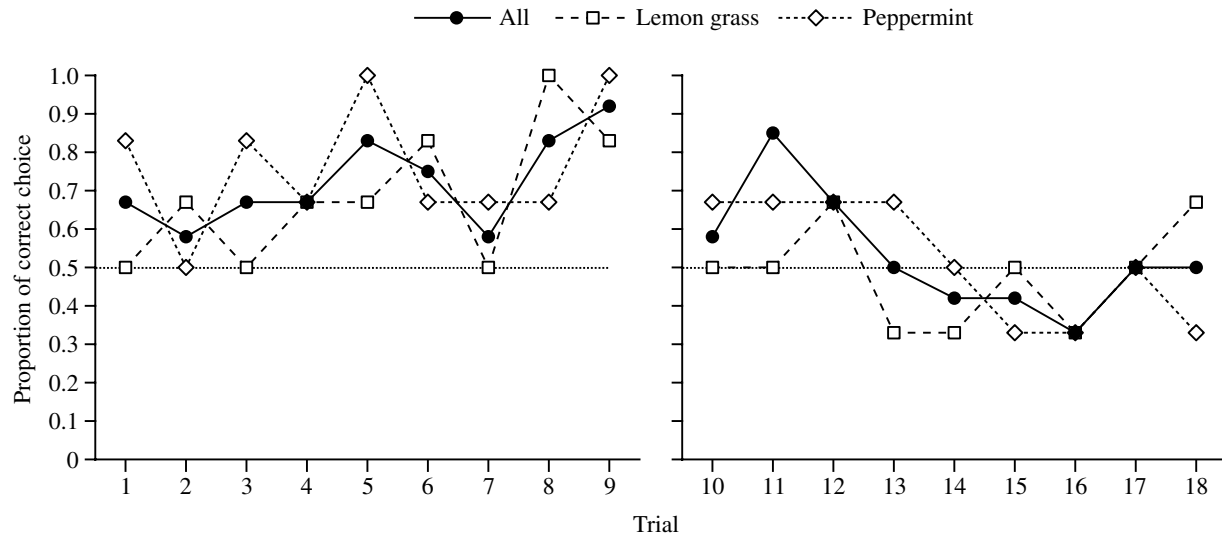


Fig. 4. Choice data, expressed as a proportion, for each of 18 successive choice-trials. Naïve locusts were allowed to choose between two scented arms (lemon grass or peppermint), one containing four pieces of wheat, the other containing one piece of wheat. A total of 12 locusts was observed, and for each locust the odour/reward combination remained constant for the first nine trials (six locusts had lemon grass paired with the high reward; six had peppermint paired with the high reward). At the 10th trial, the odour/reward combinations were reversed. Data are also presented for the positive rewards combined. For choice-trials 10–18, choosing correctly would be indicated by small values. The dotted line indicates indifference to the positive and negative rewards.

(negative or positive), but locusts in the yellow arms were more reluctant to leave the positive arms compared with the negative arms.

During the non-rewarded preference (or extinction) test, we recorded the number of entries into and the total time spent in each coloured arm. For the first analysis, in which entries were expressed in terms of percent visits to the coloured arms that previously contained the positive (larger) reward, we found locusts showed no preference [ $t_{0.05(2),8}=1.76$ ,  $P=0.117$ ]. On average, green-positive locusts made  $41.8 \pm 4.6\%$  of their visits to green arms, while yellow-positive locusts made  $58.8 \pm 8.5\%$  of their visits to yellow arms. We also found no difference between the two treatments with respect to the time locusts spent in the arm previously associated with the positive reward [ $t_{0.05(2),8}=1.20$ ,  $P=0.264$ ]. Green-positive locusts spent  $20.7 \pm 7.4\%$  of their time in green arms, while yellow-positive locusts spent  $34.2 \pm 8.4\%$  of their total time in the yellow arms. The remaining time was spent either in the other coloured arms or in the middle of the arena.

#### Experiment 2: testing odour and amount discrimination with reversal

##### Identifying test subjects

The method for identifying test locusts was similar to that used in experiment 1, except that at the end of day 2 we transferred our test locusts to the test arenas, where they remained overnight, without food.

##### Arms and rewards

To generate distinctive odours, we placed a small volume (1  $\mu$ l) of an essential oil (Culpeper Ltd, London, UK), containing either peppermint (*Mentha piperita*) or lemon grass

(*Cymbopogon flexuosus*), on a small wad of cotton wool that fitted snugly into a 1.75 ml Eppendorf tube. The top and bottom of the Eppendorf tube were removed, creating an open tube, and the tube was attached to the roof of each arm (towards the back). As in experiment 1, we placed one or four pieces of wheat (negative and positive rewards, respectively) on a moistened piece of cotton wool in the centre of each arm. Likewise, over the course of an experiment, the number of wheat pieces associated with a particular odour always remained constant, and the reward/odour combination was balanced across test locusts.

##### Behavioural observations

The protocol used for the first nine runs of the odour experiment was identical to experiment 1. After the ninth trial, however, we reversed the odour–reward combinations for an additional nine trials, rather than run an extinction test. Our aim was to record how choice preference and response latency were affected by switching the odour–reward combinations.

##### Results

In the odour experiment we asked two questions: (1) what is the effect of large and small rewards on odour discriminations, which is analogous to the colour discrimination experiment, and (2) what is the effect on performance of reversing the rewards. Fig. 4 shows the proportion of choices of the odour associated with a positive reward (Trials 1–9), followed by the proportion of choices of the same odour after the original reward–odour combination was reversed (Trials 10–18). If locusts were capable of reversal learning, we would expect them to show, during Trials 10–18, an increased preference for the odour associated with the



negative reward during Trials 1–9. Locusts showed no initial stimulus bias (eight locusts selected lemon grass first, while four selected peppermint) but, as in experiment 1, they showed a strong preference for the positive stimulus over the first nine trials [ $t_{0.05(1),11}=3.92$ ,  $P=0.001$ ]. Preference for the initially positive stimulus did decrease significantly following the reversal [ $t_{0.05(2),11}=2.45$ ,  $P=0.032$ ]. There was no significant difference between choices for peppermint and lemon grass in either the pre-reversal [ $t_{0.05(2),5}=0.79$ ,  $P=0.465$ ] or the post-reversal choices [ $t_{0.05(2),5}=0.28$ ,  $P=0.788$ ].

Log latencies for enter, approach and exit time were analysed using repeated-measures ANOVA (RMA) with stimulus (positive or negative), trial and pre- and post-reversal blocks being treated as within-subject factors, and odour (lemon grass or peppermint) treated as a between-subject factor. Here, however, we have conducted two separate

analyses. The first is restricted to Trials 1–9 so that results could be compared with experiment 1, while the second includes Trials 1–18, which allows us to examine the effect of the reversal.

As seen in Fig. 5A, the locusts' entry into the arms got faster over the first nine trials ( $F_{8,80}=4.03$ ,  $P=0.001$ ). No significant stimulus effect, however, was observed ( $F_{1,10}=4.29$ ,  $P=0.065$ ), nor was there a stimulus  $\times$  trial interaction ( $F_{8,80}=1.21$ ,  $P=0.305$ ). We also failed to detect an overall odour effect ( $F_{1,10}=0.02$ ,  $P=0.883$ ), and there was no stimulus  $\times$  odour interaction ( $F_{1,10}=0.308$ ,  $P=0.591$ ). (Note: odour as a between-subject factor was not significant for either the approach or exit latencies, and for these two latencies it never interacted in a significant manner with stimulus, trials or blocks.)

Over all 18 trials, for enter time, we found a significant difference between the pre- and post-reversal nine-trial blocks

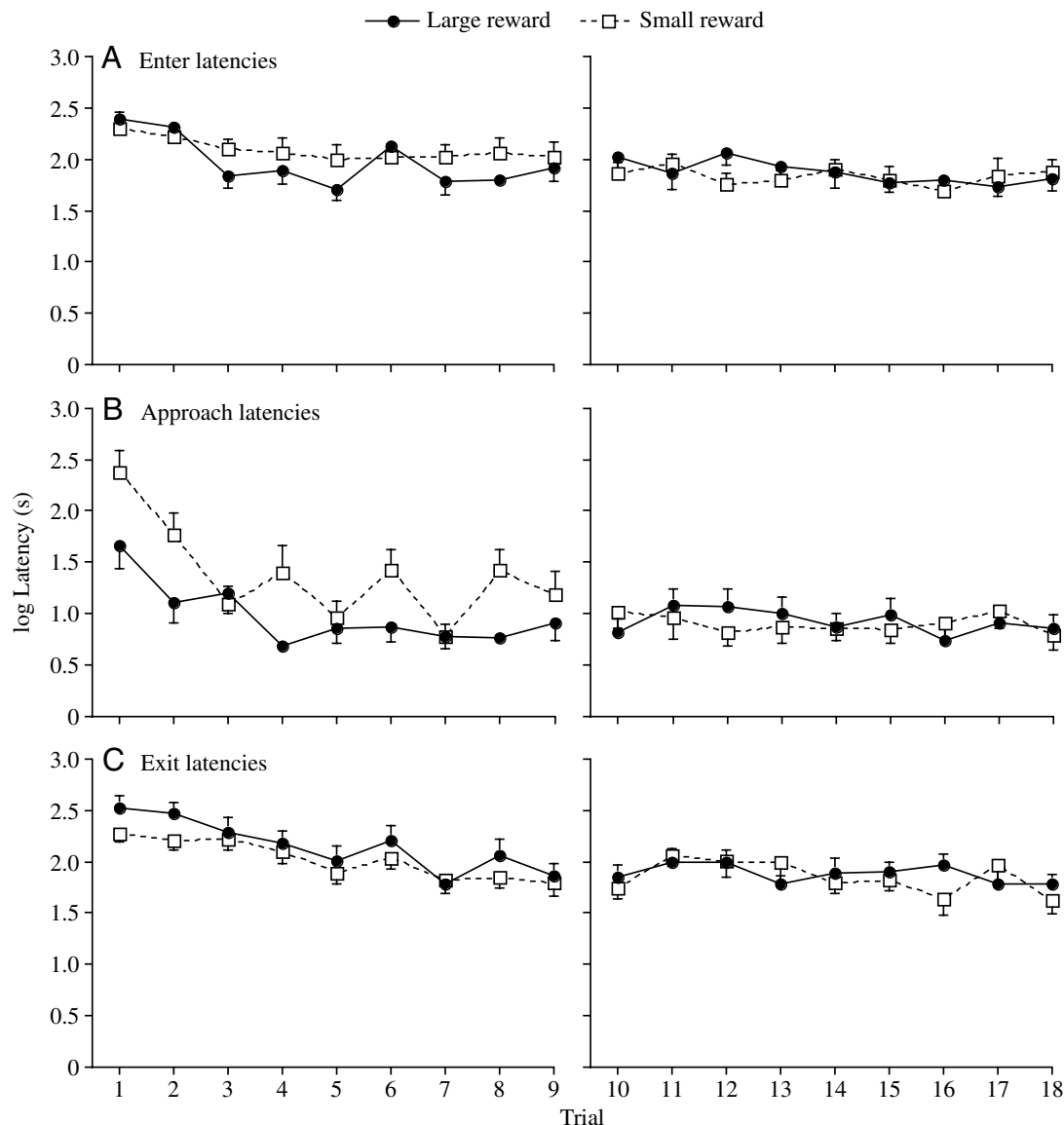


Fig. 5. The mean ( $\pm$  S.E.M.) natural log latency of response for the positive and negative odours over the course of training. At the 10th trial, the odour/reward combinations were reversed. Three measurements were recorded: (A) enter time, (B) approach time and (C) exit time.

( $F_{1,10}=11.58$ ,  $P=0.007$ ) but no stimulus effect ( $F_{1,10}=0.84$ ,  $P=0.425$ ). There was, however, a significant stimulus  $\times$  block interaction ( $F_{1,10}=5.88$ ,  $P=0.036$ ), which suggests the latency to enter a scented arm was affected by reversing reward amounts.

The approach time for the nine pre-reversal and nine post-reversal trials is shown in Fig. 5B. Within the first nine trials, we found that the overall approach time decreased with time ( $F_{8,80}=7.42$ ,  $P=0.001$ ) and that locusts approached the positive odour significantly faster than the negative odour ( $F_{1,10}=44.88$ ,  $P<0.001$ ). There was, however, no stimulus  $\times$  trial interaction ( $F_{8,80}=1.94$ ,  $P=0.066$ ). When all 18 choice-runs were analysed, a significant difference between the pre-reversal and post-reversal blocks was found ( $F_{1,10}=14.45$ ,  $P=0.003$ ), indicating that locusts' approach times got faster over training. There was an overall stimulus effect ( $F_{1,10}=19.48$ ,  $P=0.001$ ), and we detected a significant stimulus  $\times$  block interaction ( $F_{1,10}=14.32$ ,  $P=0.004$ ). The significant interaction was due to the fact that the faster approach time in the positive arm in the first nine trials was lost when the rewards were reversed in the second nine trials.

Finally, exit time is shown in Fig. 5C. In the first nine trials, there was an overall trials effect ( $F_{8,80}=7.85$ ,  $P=0.001$ ), with locusts leaving the arms faster over successive trials. We did not observe a stimulus effect ( $F_{1,10}=2.70$ ,  $P=0.131$ ) nor a stimulus  $\times$  trials interaction ( $F_{8,80}=0.57$ ,  $P=0.802$ ). We also observed a significant difference in exit time between the pre- and post-reversal blocks ( $F_{1,10}=15.53$ ,  $P=0.003$ ) and an overall stimulus effect ( $F_{1,10}=5.14$ ,  $P=0.047$ ) but no stimulus  $\times$  block interaction ( $F_{1,10}=0.69$ ,  $P=0.427$ ). On average, locusts left the arms faster in the post-reversal block (Trials 10–18) compared with the pre-reversal blocks (Trials 1–9) and, averaged over all 18 trials, they stayed in the positive arms longer than in the negative arms.

### Experiment 3: testing odour and amount discrimination with synthetic foods

The aim of this experiment was to test whether locusts could differentiate between two rewards based on differences in nutrient concentrations. Here, we used a synthetic diet, which allowed us to control both the ratio and concentration of nutrients in our test foods.

#### Test food

Dry synthetic chemical defined foods, similar to those developed by Dadd (1961) and modified by Simpson and Abisgold (1985), were made that varied in their ratio of protein (p) to digestible carbohydrate (c). Ratios (in % dry mass) were as follows: p7:c7, p14:c28, p21:c21 and p28:c14. Previous studies with *S. gregaria* have shown the p21:c21 diet to be near optimal for growth and development (Simpson et al., 2002), while the p7:c7 diet contained nutrients in ideal ratios but in suboptimal quantities. The p14:c28 and p28:c14 diets are themselves nutritionally suboptimal but, when presented in combination, they are complementary. All the foods contained 4% essential micronutrients and all except the p7:c7 diet

contained 54% cellulose (the p7:c7 food had 82% cellulose). Digestible carbohydrate consisted of a 1:1 mix of sucrose and white dextrin, while the protein contained 3:1:1 casein:peptone:albumin. The diet was suspended in a 1% agar solution in a dry:wet ratio of 1:4 and presented to individual locusts as small cubes.

#### Identifying test subjects

The protocol differed slightly from the previous two experiments because of the nature of the food. Here, newly moulted locusts were fed one block (2 cm<sup>3</sup>) of p14:c28 food and one block of p28:c14 food (food was added or replaced several times during the day to maintain freshness and quantity) on days 0 and 1. At the end of day 1 (16.00 h), all the food was removed and the locusts were left overnight. On the following morning, locusts were given two small blocks of each food, and then 1 h later we observed individual feeding behaviour. Specifically, we identified the first two individuals to consume 7–10 total blocks of food. Once identified, these individuals were given blocks of p14:c28 and p28:c14 food until the end of the day (16.00 h), at which point the locusts were transferred to the test arenas, where they remained overnight.

#### Arms and rewards

The aim of this experiment was to determine how modifying the nutrient content of the food, as opposed to bulk amount, affected associative learning. As in experiment 2, peppermint and lemon grass were placed on cotton in Eppendorf tubes, but for this experiment we used the p7:c7 and p21:c21 food, suspended in a 1% agar solution, as our rewards (a negative and positive reward, respectively). Small blocks of food (2 mm<sup>3</sup>) were placed in the middle of the arms, but in these runs no cotton wool was used. Over the course of an experiment the nutrient content associated with a particular odour remained constant, but we balanced the reward/odour combination across test locusts.

#### Behavioural observations

The protocol used for this experiment was identical to that described for the colour experiment. We conducted a total of nine choice-trials with equated exposure to each option, culminating in a 20 min non-reinforced preference test.

#### Results

The mean proportion of correct choices in the nine choice-runs, plus the proportion of choices divided by the subjects when peppermint or lemon grass was the positive stimulus, is shown in Fig. 6. Analyses revealed that locusts showed a significant preference for the positive stimulus [ $t_{0.05(2),11}=4.42$ ,  $P=0.001$ ] and that odour did not affect choice behaviour [ $t_{0.05(2),5}=0.47$ ,  $P=0.661$ ].

The enter, approach and exit latencies were again analysed using a repeated-measures approach. We found that locusts entered the arms more quickly over the course of the nine trials ( $F_{8,80}=2.96$ ,  $P=0.006$ ; Fig. 7A), but we did not observe a significant stimulus effect ( $F_{1,10}=0.40$ ,  $P=0.542$ ) nor significant

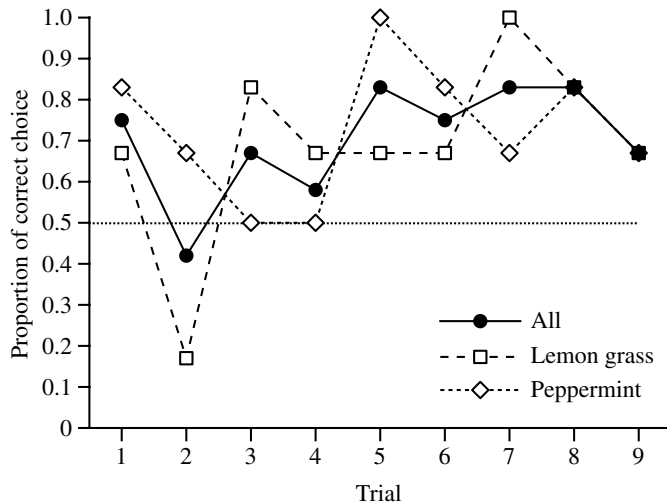


Fig. 6. Choice data, expressed as a proportion, for each of nine successive choice-trials. Naïve locusts were allowed to choose between two scented arms (lemon grass or peppermint), one containing p21:c21 food, the other containing p7:c7 food (see the Methods and Results section for a full description of these foods). A total of 12 locusts was observed, and for each locust the odour/reward combination remained constant over the entire experiment (six locusts had the high reward paired with green, while six had the high reward paired with yellow). Data are also presented for the positive rewards combined. The dotted line indicates indifference to the positive and negative rewards.

stimulus  $\times$  trial interaction ( $F_{8,80}=0.56$ ,  $P=0.805$ ). We also failed to detect an overall odour effect ( $F_{1,10}=1.34$ ,  $P=0.274$ ), stimulus  $\times$  odour interaction ( $F_{1,10}=1.13$ ,  $P=0.312$ ) or trials  $\times$  odour interaction ( $F_{8,80}=1.67$ ,  $P=0.190$ ).

The approach time is shown in Fig. 7B, and here we found a significant trials effect ( $F_{8,80}=4.01$ ,  $P<0.001$ ). The analysis also showed, consistent with the two previous experiments, that locusts approached the food more quickly in the arm associated with the positive stimulus ( $F_{1,10}=5.70$ ,  $P=0.038$ ). There was, however, no significant stimulus  $\times$  trial interaction ( $F_{8,80}=1.53$ ,  $P=0.161$ ).

The mean log latency of the exit time is shown in Fig. 7C. As in the previous two experiments, exit latency decreased over the course of the training trials ( $F_{8,80}=3.54$ ,  $P=0.001$ ). The stimulus did not affect exit time ( $F_{1,10}=0.64$ ,  $P=0.805$ ), and there was no stimulus  $\times$  trials interaction ( $F_{8,80}=1.01$ ,  $P=0.434$ ).

For the extinction test, locusts made significantly more visits to the arms holding the odour associated with the large reward [ $t_{0.05(1),10}=2.74$ ,  $P=0.010$ ]. Overall, locusts made more visits to the arms containing the odour previously associated with the large reward ( $69.7\pm 7.2\%$ ). The number of visits was not influenced by whether the positive odour was peppermint or lemon grass ( $66.4\pm 9.1\%$  and  $72.4\pm 11.4\%$ , respectively). There was, however, no significant difference [ $t_{0.05(2),10}=0.38$ ,  $P=0.856$ ] in the amount of time spent in the arms containing the odours previously associated with the positive ( $338.4$  s) and negative stimulus ( $243.8$  s).

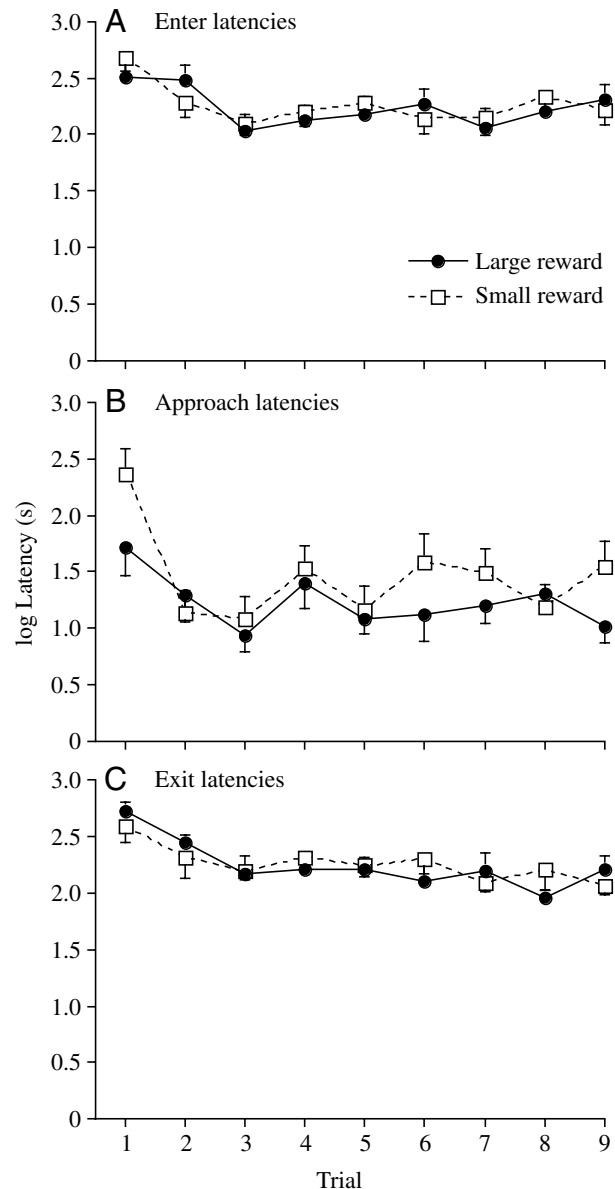


Fig. 7. The mean ( $\pm$  S.E.M.) natural log latency of response for the positive and negative odours, in response to synthetic foods, over the course of training. Three measurements were recorded: (A) enter time, (B) approach time and (C) exit time.

## Discussion

All three of our experiments are in agreement with previous work showing that acridids are capable of associative learning (e.g. Forman, 1984; Simpson and White, 1990; Raubenheimer and Blackshaw, 1994; Lee and Bernays, 1998; Behmer et al., 1999). Our study is unique, though, because it delves into the nature of associative learning in acridids by controlling levels of experience with each option, recording several measures of preference (choice, latency and extinction), examining reversal learning and documenting the change in performance during the acquisition of learning. It also reveals that acridids can discriminate between two



appetitive options, as opposed to appetitive–aversive or appetitive–neutral pairings.

Choice behaviour is generally considered a good measure of associative learning (see Bitterman, 1988, 1996) and, over the first nine trials of each experiment, locusts showed good choice behaviour with respect to both amount (experiments 1 and 2) and concentration (experiment 3) of reward. Choice behaviour was poor, however, during the reversal phase of experiment 2 (Trials 10–18), which suggests that acridids are slow to ‘unlearn’ and then ‘relearn’ odour–reward pairings. This finding is in contrast to work on free-flying insects such as the pipevine swallowtail (Weiss, 1997) and honeybees (e.g. Couvillon and Bitterman, 1985, 1986; Shapiro et al., 2001), which tend to exhibit a complete reversal of preference after 10 trials. Perhaps, with additional trials, locusts would have shown a complete reversal of preference.

Our experiments also revealed that an acridid’s response speed was sensitive to stimuli associated with greater rewards. For example, although there were no differences in latency to enter an arm from the centre chamber, once ‘surrounded’ by the stimulus (approach time), the time to reach a larger reward was significantly shorter compared with the smaller reward in all three experiments. This ‘prospective effect’ has been reported in a number of studies with vertebrates (see Goodrich, 1960; Kraeling, 1961) and in free-flying honeybees approaching colours and odours associated with a higher concentration of sucrose solution (Loo and Bitterman, 1992). The current study is, however, the first in which an insect has shown response latency differences for an option associated with different amounts of a reward (Couvillon and Bitterman, 1993). That locusts took significantly longer to exit a coloured arm associated with a higher reward (retrospective effect) is not unique to insects, having been found previously in honeybees (Loo and Bitterman, 1992).

There are, however, possible alternative explanations for our observed differences in approach and exit latencies. For instance, after locusts consumed the larger reward, they may have been more satiated and thus less motivated to seek food upon entering the arms containing the smaller rewards. Locusts are known to exhibit quiescence following a large meal (see Simpson, 1995), but in our study the larger rewards were in fact small relative to meal size during *ad libitum* feeding, so we feel a ‘satiated’ effect is unlikely. Another possibility is that time delays to the rewards may have influenced latencies. Locusts were required to ‘rest’ in the middle of the arenas for 5 min before making their first choice, but, after exiting, access to the second arm was allowed as soon as they returned to the middle of the main arena. Since locusts often chose the arm associated with the higher reward first, the time delay for entering that arm would have been, on average, greater than that for entering the arm associated with the lower reward. Latency, like choice, could be a function of associative strength, and in vertebrates such as rats, birds and fish it is considered a good measure of learning (Mackintosh, 1974). If latency is to be considered a good measure of preference in acridids, or any

insect for that matter, our protocol may require slight modifications.

Locusts, with the exception of the number of entries in experiment 3, did not score particularly well in the non-reinforced preference tests. By contrast, honeybees show strong response levels (measured as landings on nonrewarded targets) to stimuli previously associated with greater amount, concentration, probability or lower variability of reward (see Bitterman, 1996; Shapiro, 2000). This ‘resistance to extinction’ has also been a good indication of preference in birds, as measured by cumulative numbers of pecking to a non-rewarding key (M. S. Shapiro, A. Kacelnik and S. Siller, manuscript in preparation). While an underlying biological reason might explain why locusts performed poorly in the non-reinforced tests, the results may also reflect problems with our protocol. Perhaps our locusts were not given enough time to show preference differences. For example, Raubenheimer and Tucker (1997) used a non-rewarded preference test that lasted 1 h as the sole measure of preference. An interesting fundamental difference might exist between acridids and honeybees, particularly with respect to performance in non-reinforced tests. Resolving whether this is a perception, learning or performance effect may require additional work.

Basic associative learning has now been demonstrated in a number of invertebrates (e.g. fruit flies, cockroaches, *Aplysia* and crabs; see Abramson, 1994, 1997), but by far the greatest amount of parametric work has been done in honeybees (Bitterman, 1996). In many respects, we found locusts to share much in common with honeybees, but we also observed some fundamental differences. While comparing and contrasting the behaviour of acridids and honeybees was one of our goals, an additional aim was to create a protocol for studying learning in an invertebrate model to address questions that are not easily studied using honeybees. For instance, since acridids (as well as other orthopteroids, such as crickets and cockroaches) are able to independently control the intake of multiple key nutrients (reviewed by Simpson and Raubenheimer, 2000; Behmer and Nes, 2003), appetitive learning in response to specific nutrients can be studied. Certainly, one of the advantages of using acridids is that they readily consume synthetic foods (e.g. experiment 3). This permits a high level of control over nutritional content of the test foods but, perhaps more importantly, it allows researchers to control the nutritional state at the time learning takes place. Such manipulations open up a number of possibilities, including exploration of state-dependent learning (Marsh et al., 2004; Pompilio and Kacelnik, in press) and risk-sensitive foraging (choices with variability in reward; see Kacelnik and Bateson, 1996). Our technique also affords great control over the animals’ experience and timing of rewards, which allows experiments on delay of reinforcement to be conducted. Delay can be imposed by making the animal wait for food or by making it travel a greater distance for reward in the presence of one stimulus compared with another. To date, there is a great amount of vertebrate literature relating delay of reward to choice behaviour, but, with the possible exception of Lee and

Bitterman's work showing that the delay of a reward may affect preference for one target over the other (Lee and Bitterman, 1990), this issue has not been explored systematically in invertebrates.

Before concluding, it is worth highlighting some methodological issues as well as commenting on the utility of our approach for comparative studies. First, an initial selection process was used in each experiment. This was done to standardise motivational and activity levels of insects both within and between experiments, not because our testing procedure only worked on a select number of individuals. The selection technique also decreased the likelihood of watching individuals that did nothing, or very little. Second, although locusts learned both colour and odour, we feel that odours are easier to work with and are, from an acridid's standpoint, biologically more relevant. Potential problems with using colours include matching luminescence, overcoming innate biases and having access to a limited spectrum. From a biological perspective, acridids tend to live in a world dominated by shades of green, so they encounter a much narrower range of colour than, for example, free-flying nectar feeders such as butterflies and honeybees. On the other hand, different plants have characteristic smells, and acridids possess an olfactory and nervous system that allows odours to be identified and coded. Thus, odours should be correlated with food types and, compared with colour, should be easier to differentiate and learn. Third, in the artificial diet experiment, locusts received p14:c28 and p28:c14 food prior to testing (days 0–2) but p7:c7 and p21:c21 food during testing (day 3). The foods presented prior to testing were individually suboptimal, but together complementary, which allowed the locusts to self-regulate nutrient intake. During the testing phase of the experiment, novel foods (in terms of nutrient profiles) were presented for two reasons. First, we wanted the locusts to be naïve with respect to the foods they encountered. Second, we wanted the test foods to differ in their nutrient concentration but not their protein:carbohydrate ratios. Finally, one of the strengths of using acridids, or any other orthopteroid, is that their developmental stage is easily determined based on wing pad size, shape and orientation. This means that comparative studies across species are possible because a reliable marker is available that allows a degree of standardisation with respect to age. Acridids and orthopteroids are also easy to sex, which means experiments can control for gender effects.

It is clear from the published literature that parametric analyses of invertebrate models of associative learning are limited almost exclusively to honeybees (Abramson, 1997). In the present paper, we have advocated extending the invertebrate species pool, because we feel it would shed light on shared processes and phyletic differences of the learning phenomena not only within invertebrates but also with vertebrates. We also believe that it would provide a better understanding of the evolution of learning (Papini, 2002) and the biological constraints on learning in simple systems. This will require not merely demonstrating associative learning in

different species but systematically investigating the effects of parameters of reward such as amount, probability, concentration of metabolites, variability and timing on different measures of performance. While the honeybee continues to be a fruitful subject, it also has certain limitations. Perhaps acridids, especially polyphagous species such as the desert locust (*Schistocerca gregaria*) or its close cousin the American grasshopper (*Schistocerca americana*), can serve this purpose.

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